Reprinted from THE LANCET, *Jan. 2nd*, 1926, *p.* 4.

ON SOME NEW METHODS, DEVICES, AND APPARATUS FOR THE ROUTINE AND THE RESEARCH LABORATORY.

BY SIR ALMROTH E. WRIGHT, M.D., F.R.S.,

PRINCIPAL OF THE INSTITUTE OF PATHOLOGY AND RESEARCH, ST. MARY'S HOSPITAL, LONDON, W.

OF recent years a signal change has come over the spirit of medicine. It is not very long ago since the thoughtful physician believed that the methods of clinical observation and physical exploration would conduct him to his goal, and believed further that it was no part of his office to explore in his patients the biological events which lie outside the ken of our unaided senses. In contrast with his predecessor, the physician of to-day realises that direct observation and physical exploration cannot lead him far, and that he must, for the understanding of his cases, rely very largely upon *laboratory methods*—meaning here by *laboratory methods* all procedures, whether applied in the laboratory or ward, which render manifest things that are imperceptible to our unaided senses.

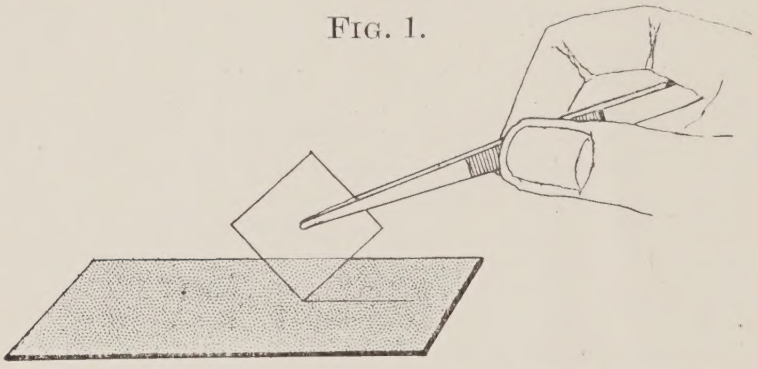
Medical research has passed through a precisely similar evolution. The earlier research worker was content to restrict his observations to the data furnished by the unaided senses. Pasteur, for example, in his immunisation work, took cognisance only of phenomena such as the survival, sickening, and dying of inoculated animals. In other words, he was content to employ the method of the *saltus empiricus*—the method by which we overleap all intermediate and "*subperceptible*" events to a final result which becomes visible to the eye. Since that the research worker has been learning his lessons. He has learned that only very few problems can be resolved by the method of direct observation. He has learned, further, that problems which have been resolved by this method (let us think here, for instance, of small-pox vaccination) have been only quite superficially resolved. And he has learned, lastly, that the intimate mechanism of things cannot be laid bare except by resort to appropriate laboratory methods.

Herein lies the justification for the publication of new laboratory methods, devices, and apparatus. Inasmuch as it is this latter which must first be taken into account, the description of the procedures in the following will in each case be prefaced by explaining how the necessary apparatus is made.

Cover-glass Cells.

For the proper display and enumeration of such elements as red and white corpuscles, or microbes, or bacterial colonies growing in fluid, we require in each case to have at disposal shallow cells of standard depth. The hæmocytometer, of course, provides such a cell, and so does the slide-cell described in a previous paper.[1] The hæmocytometer has the grave defect of being expensive. Further, in the hæmocytometer cell the depth of fluid cannot be altered to suit different requirements. And, again, the hæmocytometer cell does not lend itself to sterilisation and to conversion into a culture chamber such as is required in connexion with measurements of bactericidal power. The slide-cell, though it provides ideally convenient culture chambers, falls short in the respect that its lid, made as it is of a microscopic slide, does not allow of the examination

Fig. 1.



Showing how the edges of a cover-glass are coated with vaseline by trundling it over the surface of a hot vaseline-covered slide.

of the contents of the chambers with high magnifying powers. With a view to remedying these defects of the hæmocytometer and the slide-cell the cover-glass cell was devised. It is designed upon the very simple principle that a known volume of fluid spread out under a cover-glass of known area will, if the escape of fluid from under the cover-glass is prevented, give a layer of fluid of known and (assuming that the cover-glass and slide are nearly flat) of approximately uniform depth.

The Simple Cover-glass Cell.

Here the escape of fluid from under the cover-glass is prevented by coating the edges of the cover-glass with vaseline. The device for applying the vaseline to the edges of the cover-glass is to place a very small pellet of vaseline upon a slide and then melt this down by heating the glass. While the vaseline is still liquid we grasp a cover-glass in a pair

[1] The Lancet, 1923, i., 365.

22500888019

of fine forceps and trundle it upon its edge over the surface of the vaselined slide in the manner indicated in Fig. 1. When a cover-glass thus edged with vaseline is imposed on a drop of fluid we have made what we may call a *simple cover-glass cell.*

Hinged and Walled Cover-glass Cell.

A second form of cell—better in the respect that it can be used both as a counting and a culture chamber —is the following.

Method of Construction.—A microscopic slide is carefully cleaned and is passed through the flame to sterilise it. A cover-glass is similarly prepared. The latter is laid down upon the slide. With a soft paint-brush melted vaseline is now painted round its edges, the vaseline being allowed to overlap the edges to the extent of a millimetre or two. One of the corners nearest the worker is left unvaselined to facilitate lifting the cover-glass (Fig. 2, A). We further require to make a hinge upon which the cover-glass can be flapped up and down without lateral dislocation. That hinge is provided by thickening the vaseline rim and allowing it to overlap a little more along the edge which is farthest from us.

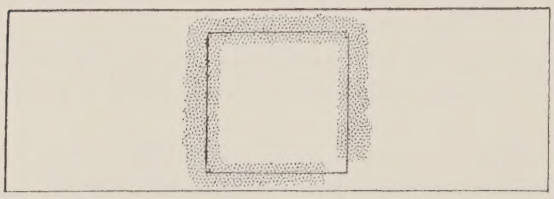
When the time comes for filling the cell a thin knife blade is inserted under the bare corner of the cover-glass lid, and this is lifted up until it stands upright upon the hinge that has been constructed (Fig. 2, B). The prearranged volume of fluid is then filled in from a pipette which has either been paraffined at the tip or has been drawn out into a fine point. If the fluid in question is blood, or any suspension in which the solid particles bulk as largely as they do in blood, not less than 30 c.mm. should be filled into the $\frac{3}{4}$-inch cover-glass cell, so as to provide a sufficient depth of fluid, and the fluid should be deposited, not as a solid drop in the middle of the floor, but in the form of a ridge running out at right angles from the middle of the hinge. If these precautions are observed and the cover-glass lid is let down very slowly upon the fluid, an uneven distribution of the corpuscles or other suspended material will be avoided. When the cover-glass has been let down upon its bed the cell is sealed up by painting additional vaseline round its edges (Fig. 2, C) We may call the sort of cells we have been here speaking of *hinged and walled cover-glass cells.*

The simple cover-glass cells may be employed as *counting chambers* for the enumeration of red and white corpuscles or bacteria.

The hinged and walled cells are used: (*a*) As *bacterial culture chambers.* Such chambers find useful application: (*a*) when we want to count the living, as distinguished from the total bacterial content of a culture, or to count the living microbes in a quantum of culture used for a bactericidal

experiment; (*b*) where we want to discover whether a particular microbe can cultivate itself in blood or any other medium; or to determine whether an addition, and if so, what addition, of a vaccine or other chemical agent will impede or abolish such growth.

FIG. 2, A.

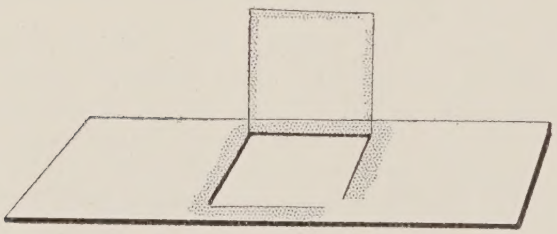


Vaseline hinged and walled cover-glass cell. The cell before opening.

(*b*) As *emigration chambers* — for the procuring of cover-glasses carpeted with, or of cells covered roof and floor with, leucocytes obtained direct from the blood.

The methods of employing the cover-glass cell for these several purposes may now be very briefly described.

FIG. 2, B.

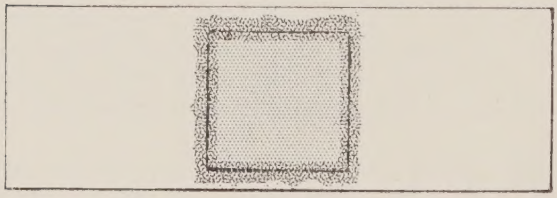


The cell opened for filling, showing the cover-glass standing upright upon the vaseline hinge.

Enumeration of the Red and White Corpuscles.

(1) Blood suitably diluted with 8 per cent. magnesium sulphate coloured with methyl violet is introduced into the cell in the manner previously described. The appropriate magnesium solution is a saturated solution five-fold diluted to which about one-tenth of a 1 per cent. solution of methyl violet in water has been added. For counting leucocytes a dilution of 1 in 50 of blood and for red corpuscles one of 1 in 500 will give convenient counts. These dilutions are made on a paraffined slide with a paraffin-tipped pipette in the manner described in the author's "Technique of the Teat and Capillary Tube."[2] A suitable cover-glass to use is one

FIG. 2, C.



The cell reclosed after filling with blood.

[2] London: Constable and Co. 1921.

20 mm. ($\frac{3}{4}$ inch) square—i.e., one with a superficial area of 400 sq. mm. Forty c.mm. of the diluted blood may be put into a cell of this size, giving a depth of 0·1 mm. If the cover-slip is not exactly 20 mm. square a correction must be applied in the calculations. When the above-mentioned quantum of diluted blood has been measured into the cell the lid is closed down, and the preparation is now placed under the microscope fitted with either a high-power dry or an oil-immersion lens. The diameter of the field which this lens gives when employed with the selected eye-piece must have been previously determined by measurement with a stage micrometer. For the purpose of an enumeration the number of cells red or white in, let us say, 10 or 20 fields is ascertained and an average is then struck.

The calculations come out very simply. Let us suppose, for the sake of illustration, that our data were as follows. *Diameter of the field of vision of our lens combination*, 0·8 mm. *Superficial area of that field of vision* (arrived at by squaring the diameter and multiplying by 0·785), 0·5 sq. mm. (approximately). *Depth of fluid in the cell* (supposing 40 c.mm. of fluid to have been filled into a cell of 400 mm. superficial area), 0·1 mm. *Volume of diluted blood in the field of the microscope*, 0·05 c.mm. *Volume of undiluted blood in the field of the microscope* (assuming the blood to have been diluted 50-fold), 0·001 c.mm. And now, assuming that the average number of leucocytes per field had worked out as 7, the figure for the number of leucocytes in 1 c.mm. of blood would work out as 7000.

The method of calculation is similar for a red corpuscle count, except that here allowance must be made for the ten-fold greater dilution of the blood. When the cell is used for the enumeration of the microbes the procedure is similar to that used for counting corpuscles.

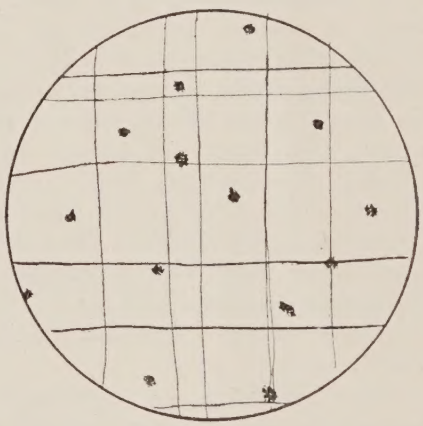
Procedure for the Enumeration of Microbes.

(2) Progressive dilutions of the culture are made (salt solution, broth or diluted serum being used as the diluent) and then the most suitable dilution (it will be one which furnishes only a very few microbes in the field of the oil-immersion field) is chosen for enumeration. Certain special difficulties, however, present themselves when we try to focus down upon small unstained objects when present in only very small numbers in a water-clear medium. Let us carry in mind here that we have in the oil-immersion lens the equivalent of an extremely myopic eye which possesses only a very shallow depth of focus. Further, let us reflect that when we are focusing down upon

a preparation such as we have here in view there is nothing to give us warning (unless by special providence a microbe happens to lie in the exact axis of vision, and that does not count as adequate warning); there is nothing, I resume, to notify us of our having arrived at the upper delimiting plane of our fluid, and again nothing to give us warning of our having arrived at the bottom of our layer of fluid. In default of such guidance we can hardly fail to focus either too high or too low. And focusing too high means failing to see our microscopic objects, and focusing too low means in addition ruining our preparation.

These difficulties can be overcome by the adoption of a very simple device. We take a piece of very fine emery paper (the kind that is described in the trade as 00), and making firm pressure upon this draw it once longitudinally over our microscopic slide. That done we take our cover-glass and lay it down upon the emery paper, and pressing firmly down upon it with the finger, move it over the paper for the distance of about an inch. This gives us two sets of parallel striæ —one upon the upper surface of the slide, the other on the lower surface of the cover-glass. We now in constructing our cell impose the striated face of the cover-glass upon the striated face of the slide, taking care to dispose the striæ on the cover-glass at right angles to those on the slide (Fig. 3). As we focus down upon such a cell, the vertically disposed scratches on the cover-glass will, of course, come first into view, and on focusing down further the longitudinal striæ on the slide. Between these two delimiting planes we shall find our microbes. Our counting will be facilitated if we use only a small depth of fluid in our cell. Not more than 20 c.mm. of fluid should be employed for a 20 mm. square cell. And it is convenient to use even less than this, provided always that this can be done without entangling bubbles in the fluid. In point of fact the avoidance of these is much facilitated by the circumstance that we are here operating not with smooth but with scratched glass surfaces.

FIG. 3.



Microbic colonies in the field of the microscope, enclosed between two systems of striæ —the vertical striæ being on the lower face of the cover-glass and the horizontal on the upper surface of the slide.

Employment of the Cover-glass Cell as a Culture Chamber.

(3) When the cover-glass cell is used as a culture chamber for counting living microbes it is, of course, requisite to employ a nutrient medium in which the microbes can grow out rapidly into colonies. When dealing with serophytic bacteria we may conveniently employ serum, or diluted serum or blood diluted with three times its volume of water. When the culture in progressive dilutions has been implanted into one or other of these media, appropriate volumes are measured into a series of cover-glass cells and these are sealed up and placed in the incubator. After they have been there for 24 hours they are taken out and examined. When a sufficient depth of nutrient fluid has been employed in the cell (for example, when 30 or 40 c.mm. of fluid have been introduced into a $\frac{3}{4}$-inch cover-glass cell) the colonies when few in number will be sufficiently large to count with the naked eye. When the colonies are more numerous they can be enumerated with a hand-lens—the enumeration being facilitated by ruling with a grease pencil a pattern of squares upon the lower surface of the slide. When the colonies are more numerous still, or when they have remained very small (as will happen when only a very shallow layer of nutrient medium has been provided) (Fig. 3) enumeration is carried out under the microscope by the procedure which has been described in connexion with red and white blood corpuscles.

Inasmuch as the cover-glass cell in the capacity of a culture chamber finds application in particular in connexion with measurements of the bactericidal power of the blood, the procedure for making these measurements may be very briefly described. It consists essentially of two operations. In the first, measured volumes of the diluted culture are added to blood and then in each case aliquot portions of this implanted blood—conveniently 40 or 50 c.mm.—are introduced into a series of cover-glass cells—taking care when introducing the blood into the cells to spread it out, as described above, in the form of ridge at right angles to the middle of vaseline hinge. The cells are then sealed and placed in the incubator. In the second operation the self-same quantities of culture are implanted into either serum or hæmolysed blood, and again similar volumes of these media are introduced into another series of cover-glass cells, and are placed in the incubator. And, finally, after 24 hours the colonies in the companion cells are counted, and the results are expressed in the form of proportion sums.

Employment of the Cover-glass Cell as an Emigration Chamber.

(4) The cover-glass cell furnishes also a very convenient emigration chamber—in other words, we

can employ it for obtaining a cover-glass carpeted with living leucocytes, or if we happen to want this a cell which is both floored and roofed with these elements. All that requires to be done is to fill the cell with blood—60 c.mm. is a convenient quantity—and to impose a cover-glass upon the blood while it is still unclotted. The cell is now placed in the incubator for a shorter or longer period ; for a shorter period, if we want a thin carpeting consisting exclusively of polynuclears ; for a longer, if we want a thick carpeting with an admixture of mononuclears. For the former purpose 20 to 30 minutes ; for the latter 45 minutes will amply suffice.

Method of Obtaining a Permanent Record of Bactericidal Power of Blood.

When it is for any reason desirable to keep a permanent record of measurements of the bactericidal effect exerted by a sample of blood the following requirements must be satisfied. The blood with its implanted microbes must be incorporated into some texture which will keep these micro-organisms separate, and will allow of the survivors growing out in its interstices. Further, the containing texture must be one in which the colonies can be rendered conspicuous by staining while it itself fails to hold the stain. These conditions are fulfilled by filter paper, and upon it colonies of staphylococcus or streptococcus, stained by Gram's method (or of tubercle bacillus stained by Ziehl-Neelsen), stand out in good contrast. Care must be taken with the sterilisation of the filter paper. This should be carried out by heating in the dry steriliser for ten minutes at 120° C. If a longer time or a higher temperature is employed the absorbent properties of the paper may be impaired.

Method of Procedure.

The procedure is as follows. We take a measured volume of serum, plasma, or blood (100 c.mm. is a suitable amount) and mix with it on a paraffined slide a measured volume of a suitable dilution of a microbic culture. Into this implanted blood, plasma, or serum we now immerse a disc of sterile filter paper. When it has impregnated itself with the fluid we transfer it to a sheet of sterile paper and so get rid of any excess of fluid. That done, we transfer the disc to the surface of a sterile slide, cover it with a sterile cover-glass, then paint melted vaseline round the edges so as to prevent desiccation, and then put away for 24 hours (or if we are dealing with the tubercle bacillus for a week) into the incubator. After that the cover-slip is removed and the specimen is placed in water, the soaking being continued until the albuminous substances of the blood fluids have

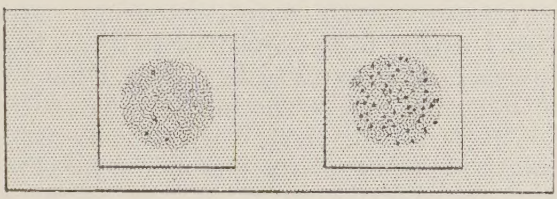
soaked out and the hæmoglobin has been completely extracted. We then, in the ordinary case where we are dealing with either staphylococcus or streptococcus, stain by Gram's method, continuing the decolorisation until the colonies stand out as dark violet spots upon a perfectly white background. Where we are dealing with the tubercle bacillus we, of course, stain with boiling carbol-fuchsin and decolorise with sulphuric acid. Finally, in either case we dehydrate in absolute alcohol, then remove the alcohol by pressure between filter paper, soak for a minute in xylol, and then mount in Canada balsam (Fig. 4).

Method of Removing Leucocytes from Blood.

The emigration cell, described in a previous section, renders service by providing us with isolated leucocytes obtained from the normal blood such as are required for the investigation of the bactericidal power of these elements.

In order to obtain counterpart experiments with blood deprived of leucocytes, it was requisite to find means to remove the leucocytes from blood. It turned out that this can be very simply achieved by passing defibrinated blood through even one thickness of filter paper. This filtration procedure furnishes, in addition to a supply of filtered blood, also filter paper whose upper surface is crowded, and whose lower surface is almost bare of leucocytes. Thus such filter papers furnish us with the means of making comparative bactericidal experiments with what we may call *hyper-* and *hypo-leucocyted* blood—i.e., blood which is very rich, and blood which is very poor in leucocytes.

Fig. 4.



a *b*

Showing microbic colonies stained in filter paper, the filter paper being impregnated in *a* with hyper-leucocyted in *b* with hypo-leucocyted blood.

To conduct such experiments we take discs or squares of sterilised filter paper, impose them upon a paraffined slide, and then using the heated handle of a metal scalpel as a flat-iron we iron down the edges of the papers upon the slide in such a manner as to impregnate these edges with paraffin. The border of paraffin which is in this way obtained serves to keep the blood when this is imposed upon the filter discs or squares from running off upon

a subjacent surface. Our paraffin-framed discs or squares are now transferred to a sterile sheet of filter paper which is destined to supply the aspirating force required for our filtering operations. Everything being now prepared, we impose defibrinated blood upon our filters heaping it up upon them, and we now take care to bring the upper and lower filter papers into closer application so as to start the filtration. In filtering care must, of course, be taken to prevent any portion of the filter running dry.

That a filter paper which has served for the filtration of blood contains in its superficial layers practically all the leucocytes of the blood, and in its deeper layers practically no leucocytes can be very easily demonstrated. We can satisfy ourselves of this by taking such a filter paper, treating it with water until all the hæmoglobin has been dissolved out, staining with oil-imod, mounting in balsam, and examining under an logwomersion lens.

For the purpose of a comparative bactericidal test we prepare two such discs (Discs Nos. 1 and 2), also soak another precisely similar disc in serum (Disc No. 3), and then take a Petri dish containing nutrient agar, the surface of which has been evenly implanted with staphylococci. Then we place Disc No. 1 upper face downwards upon the agar ; Disc No. 2 lower face downwards ; and Disc No. 3 any way that it happens to come, and then cultivate at 37° C. for 24 hours. By that time all the microbes which have escaped destruction will have grown out into colonies, and will have invaded the substance of the filter paper discs, and inspection of these, beginning with the last and ending with the first mentioned, will show (1) that the serum has exerted no bactericidal power. (2) that the hypo-leucocyted blood has exerted very little bactericidal effect, and (3) that the hyper-leucocyted blood has exerted a very great deal. Permanent show specimens can now be prepared by first soaking the filter papers in water, then staining by Gram's method, and then mounting in Canada balsam (Fig. 4).

The Vaginal Pipette.

The vaginal pipette serves for evacuating the contents of lengths of capillary tube which are unprovided with mouthpieces—for example, stems of capillary pipettes which have been cut off from their butt ends.

The principle upon which the pipette is constructed will be understood from the description of the method of making such a pipette. We take a capillary pipette with tapering stem, and cut this down to a length of about 3 inches, and then again cut it across just below the neck of the pipette. We then coat the middle of the truncated stem somewhat heavily with sealing-wax, and heating the neck of the

butt end in the flame we bring it down over what was previously the distal extremity of the capillary tube until it meets the sealing-wax. This lutes the two tubes together, giving us an inner tube with a trumpet-like aperture and a tapering upper end, and an outer tube upon which we can fit a rubber teat (Fig. 5). When we now introduce into the pipette's trumpet-shaped mouth the tapering end of any open capillary stem, and push it home until grasped by the contracting walls of the tube, we obtain a practically air-tight joint. When such an air-tight joint has been obtained pressure on the teat will expel the contents.

FIG. 5.

FIG. 5.—Vaginal pipette.

FIG. 6.

FIG. 6.—Coagulation tubes fixed on a roll of plasticine. The left-hand two are depicted empty except for the paraffin lining in their upper tapering ends. The right-hand two are shown with the columns of blood in position.

This description of the vaginal pipette may conveniently introduce an account of two procedures in which it finds useful application. The first of these is a

Simplified Procedure for Measuring the Phagocytic Power of the Blood, or, as the case may be, the Phagocytic Efficiency of Leucocytes, or the Opsonic Index.

The technique here to be described departs from that set out in the author's "Technique of the Teat and Capillary Tube" in the respect that after the phagocytic mixtures have been made and filled into the capillary pipettes and the tips of these have been sealed, the

stems are cut off from the butt ends and are then immediately or after a very short incubation in the water-bath centrifuged. After a centrifugalisation, which may last on the average some 45 seconds, the tips of the pipettes are cut off, the contents are expelled and mixed by the aid of the vaginal pipette, and films are made in the customary manner. In a future communication the details and the advantages of this technique will be fully described.

Simplified Procedure for Measuring the Coagulation Time of the Blood.

The method here to be described is a simplification of the methods described in the first and second editions of the author's "Technique of the Teat and Capillary Tube." We take a number of capillary tubes of approximately equal calibre and dip their distal ends into thoroughly liquefied paraffin wax, allowing this to run up the tubes to a distance of about 1½ inches. The bulk of the paraffin is then blown out, leaving us with a set of capillary tubes whose tapering distal ends carry a lining of paraffin. We now cut off the stems some 2 to 3 inches above the upper border of the paraffin lining (Fig. 6). The tubes—let us now call them *coagulation tubes*—are then arranged palisade fashion, but not too closely together, upon a roll of plasticine with their paraffined ends disposed upwards and projecting both a little above and a little below the plasticine. Blood is then obtained by puncturing the finger and is allowed to drop on to a paraffined slide. The non-paraffined ends of the coagulation tubes are now approximated and are dipped into the blood. When this has run up each tube to the distance of about 1½ inches the inflow is arrested and the tubes are inverted so as to allow the blood to gravitate down in the direction of the tapering paraffined ends. The tubes are now immersed, paraffined ends upwards, in a jar of water standing at 37° C., the plasticine roll to which they are connected being affixed to the side of the jar just above the water. It will now be found that the paraffined lining as applied to the tapering end of the tube effectively counteracts the upward pressure of the water with the result that the columns of blood now remain fully immersed. When the time for investigating the condition of coagulation in each successive tube arrives the contents of that tube are, by the aid of the vaginal pipette, blown out into the surrounding water.

WELLCOME INSTITUTE LIBRARY
Coll. wellcome
Coll. pam
No. W 20
1926
W 94 o

The Lancet Office,
1, Bedford Street, Strand, W.C.2.